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Cell Injury, Repair, Aging and Apoptosis

Tissue Transglutaminase Contributes to Interstitial Renal Fibrosis by Favoring Accumulation of Fibrillar Collagen through TGF-β Activation and Cell Infiltration

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Renal fibrosis is defined by the exaggerated accumulation of extracellular matrix proteins. Tissue transglutaminase (TG2) modifies the stability of extracellular matrix proteins and renders the extracellular matrix resistant to degradation. In addition, TG2 also activates transforming growth factor-β (TGF-β). We investigated the involvement of TG2 in the development of renal fibrosis using mice with a knockout of the *TG2* gene (KO). These mice were studied at baseline and 12 days after unilateral ureteral obstruction, which induced a significant increase in interstitial TG2 expression in wild-type mice ($P < 0.001$). Interstitial fibrosis was evident in both groups, but total and fibrillar collagen was considerably lower in KO mice as compared with wild-type ($P < 0.001$). Similarly, mRNA and protein expression of collagen I were significantly lower in KO animals ($P < 0.05$). A statistically significant reduction in renal inflammation and fewer myofibroblasts were observed in KO mice ($P < 0.01$). Free active TGF-β was decreased in KO mice ($P < 0.05$), although total (active + latent) TFG-β concentration did not differ between groups. These results show that mice deficient in TG2 are protected against the development of fibrotic lesions in obstructive nephropathy. This protection results from reduced macrophage and myofibroblast infiltration, as well as from a decreased rate of collagen I synthesis because of decreased TGF-β activation. Our results suggest that inhibition of TG2 may provide a new and important therapeutic target against the progression of renal fibrosis. ***(Am J Pathol 2008, 173:631–642; DOI: 10.2353/ajpath.2008.080025)***

Worldwide, increasing numbers of patients are affected by chronic kidney diseases. Renal fibrosis is the final common pathway of a wide variety of chronic kidney diseases, irrespective of the initial causes of nephropathy. It is defined as an excessive accumulation and deposit of extracellular matrix (ECM) components, leading to complete destruction of kidney tissue and renal failure.[1] Different approaches have been proposed to combat renal fibrosis: (i) decreasing ECM protein synthesis, (ii) promoting ECM protein degradation, or (iii) preventing mesangial and fibroblast activation and tubular epithelial-mesenchymal transition. Among the many profibrotic factors involved in renal fibrosis, angiotensin II and transforming growth factor-β (TGF-β) play a central role.[2,3] However, despite of a maximum blockade of renin-angiotensin-aldosterone system with combination of ACE inhibitor, AT-1 antagonist and diuretic, renal function still declines, emphasizing the need of new therapeutic targets.[4]

So far, little attention has been paid to post-translation modification of ECM proteins by regulating enzymes. Excessive accumulation of ECM can be triggered by ECM-regulating enzymes. Tissue transglutaminase (TG2) is a calcium-dependent enzyme that catalyzes post-translation

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modification of proteins through acyl transfer reaction between the γ-carboxide group of a peptide-bound glutamyl residue and various primary amines.[5] It is constitutively expressed in endothelial and smooth muscle cells, whereas in other cell types it is induced by distinct signaling pathways. The enzyme plays a role in celiac disease, neurodegenerative diseases, and tissue fibrosis. TG2 contributes to fibrogenic processes, including atherosclerosis, and lung, liver, and renal fibrosis, by different mechanisms.[6] Extracellular TG2 has the capacity to irreversibly crosslink matrix proteins including collagens, fibronectin, laminin, nidogen, and proteoglycans,[7] leading to increased ECM resistance to proteolytic enzymes and deposition.[8] In addition, TG2 can favor fibrosis by contributing to TGF-β1 activation. This activation requires a multiple-step process ending with the release of a free active TGF-β, able to bind and activate its receptors.[9] Independently of its crosslinking activity, TG2 interacts with integrins and provides a binding site for fibronectin, and thus serves as a co-receptor, facilitating adhesion, spreading and motility of cells.[5,6] Intracellular expression of TG2 has been associated with cell death and clearance of dying cells.[10]

Previous studies proposed that TG2 could be involved in fibrotic diseases. Cardiac overexpression of TG2 resulted in diffuse cardiac interstitial fibrosis with depressed ventricular function.[11] Regarding the kidney, TG2 expression and tissue level of ε(γ-glutamyl) lysine crosslink were increased in subtotal nephrectomy and in unilateral ureteral obstruction (UUO) models,[12,13] and recent studies by Johnson et al[14] indicated that global transglutaminase inhibition by pharmacological agents reduced fibrosis and preserved function in the five-sixths subtotal nephrectomy model. In humans, a strong association between renal fibrosis and function with the expression of TG2 and its crosslink product was observed irrespective of original nephropathy.[15–18]

To date, it is unknown whether absence of TG2 can prevent and/or decrease renal fibrosis. This study investigated the effect of absence of TG2 on the development of interstitial fibrosis induced by UUO, using mice lacking the expression of TG2. Our results show that TG2 knockout (KO) mice were protected against the development of renal interstitial fibrosis, which was associated with a lesser activation of TGF-β1 and reduced interstitial inflammation.

Materials and Methods

Animals and Experimental Design

Male TG2 KO C57BL/6 mice generated and described previously[19] and age-matched WT controls underwent UUO or a sham-operation (ureter manipulated but not ligated). Surgery was performed under general anesthesia after intraperitoneal injection of pentobarbital sodium 55 mg/kg. The left ureter was ligated at two separated points in the UUO group through a left flank incision. Mice were allowed to recover for 12 days before tissue collection. Animal numbers for each group are outlined in each section. All animal procedures were in accordance with European Union Guidelines for the Care and the use of laboratory animals and were approved the local ethic committee.

Baseline Studies

Measurement of Blood Pressure and Heart Rate

Systolic arterial pressure and heart rate were measured with a tail-cuff sphygmomanometer adapted to the mouse, using automated system (MC 4000 BP analysis system, Hatteras Istruments, Inc. Cary, NC). To avoid variations in blood pressure due to day cycle, all measurements were performed between 9 and 11 AM. Animals were accustomed for several days before measurements. Only animals that did not display signals of stress and that showed stable and reproducible values of blood pressure for at least three consecutive days were considered for blood pressure measurements. Ten measurements from each mouse were taken at 2-minute intervals, and a mean value was determined. Systolic arterial pressure is expressed as mm Hg and HR as beat per minute.

Measurement of Urinary Albumin Excretion

All mice were acclimated in metabolic cages with free access to food and water for 24-hour urine collection. Measurements of microalbuminuria were performed using the Olympus System Reagent (ref OSR6167) and an Olympus AU 400 apparatus. Urinary albumin concentration was normalized to urinary creatinine concentration, and values were expressed as mg albumin/mmol creatinine.

Measurement of Glomerular Filtration Rate

Glomerular filtration rate was measured using inulin clearance as described previously.[20] Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and placed on a servo-controlled heating table that maintained body temperature at 37°C. A tracheotomy was performed, and a tracheal polyethylene (PE-60) catheter was inserted to facilitate breathing. The right femoral vein was cannulated with a pulled PE-10 catheter for continuous infusion of isosmotic saline solution (0.9% NaCl, 10 μl/min) and bovine serum albumin (2.5%) with Inulin conjugated to fluorescein isothiocyanate (FITC) (10 μl/min). This infusion rate was selected to ensure body volume homeostasis. Additional doses of pentobarbital were given intravenously as required. The right femoral artery was cannulated with a tapered PE-100 catheter connected to a pressure transducer for continuous monitoring of systolic, diastolic, and mean arterial pressures. The bladder was cannulated with a PE-50 catheter that allowed the collection of urine.

Blood and urine were collected over a period of 20 minutes. Approximately 80 μl of blood were collected with a heparinized capillary tube. Fluorescence was determined using a spectrophotometer with 485-nm excitation and read at 538-nm emission. The relationship between fluorescence and inulin concentration was examined by measuring fluorescence in serial dilutions of a FITC-inulin solution of known concentration. Glomerular filtration rate was evaluated by calculating inulin clearance using the 20-minute urinary FITC-inulin excretion rate (urinary fluorescence counts/20

minutes) divided by the concentration of plasma FITC inulin (fluorescence counts/μl). Glomerular filtration rate was expressed as μl/min/g.

Renal Morphology and Analysis of Tubulointerstitial Fibrosis

Kidneys were removed from mice after 12 days of UUO ($n = 10$) and sham ($n = 10$) operations for structural analysis. Kidneys were cut in four equivalent parts. One part was fixed in alcohol formol acetic acid solution, then embedded in paraffin wax, and cut at 3 μm. Sections were stained with Masson's trichrome or Sirius red staining to quantify extracellular matrix. The three other parts were frozen at −80°C for total collagen content measurement, immunohistochemistry, and total RNA extraction from isolated renal cortex.

Interstitial fibrosis was assessed semiquantitatively on Sirius red-stained paraffin sections at magnification of ×20, under polarized light. Interstitial fibrosis was quantified using computer-based morphometric analysis software (Axionplan, Axiophot2, Zeiss, Germany). A specific stain pattern was selected and the percentage of positive area in the examined tubulointerstitium was measured. Ten fields were selected randomly from each kidney representing nearly the whole piece of cortex. All scoring was performed in a blinded manner on coded slides. Data are expressed as percentage of positive area examined.

Immunohistochemistry and Immunofluorescence

Myofibroblasts and epithelial to mesenchymal transition were revealed by adding a polyclonal rabbit anti-human S100A4 (1:100, Dakocytomation, Danemark) and a rabbit polyclonal anti-human E-cadherin (Usbiological, USA), respectively, on paraffin-embedded kidney tissue, incubated overnight at 4°C in a humid atmosphere. Paraffin sections were previously treated by Target Retrieval solution at low pH (Dakocytomation), at 95°C for 40 minutes. Tubular proximal cells were recognized using an anti-megalin (1:100, kind gift from P. Verroust, Paris, France[21]). Three μm thick unfixed cryostat sections were placed onto super frost glass slides (Menzel GmbH & Co KG), and fixed with acetone for 3 minutes and stored at −20°C before use. Sections were treated with peroxidase inhibitor for 10 minutes to block endogenous peroxidase activity. Then, the sections were incubated 10 minutes with avidin and biotin. Between each incubation period, sections were washed in PBS. For macrophage cell infiltration, a biotinylated Rat anti-mouse F4/80 (1:100, MCA497B, Serotec) was incubated for 1 hour. For type 1 collagen assessment, a rabbit anti-mouse collagen type 1 polyclonal antibody (1:100, AB765P, Chemicon International, Inc) was added for 2 hours. Sections were washed in PBS, incubated with streptavidin secondary antibody 1:200 dilution for 30 minutes, and washed with PBS or with Histofine kit solution for 30 minutes. 3-amino-9-ethylcarbazole were added for signal detections then sections were counterstained with Hematoxylin followed by immersion in Scott's tap water. Finally, slides were mounted with aqueous mounting medium (DAKO). All steps were performed at room temperature. Rat and rabbit IgG were used as negative controls. Immunohistochemical quantification was performed using the analysis system described above and results are expressed as percentage of positive area. For immunofluorescence staining, renal TG2 expression was studied using a Rabbit anti-TG2 antibody (997-PTG, Covalab, France) incubated at 1:100 dilution for 2 hours at room temperature, on unfixed cryostat section to detect insoluble TG2.[15] The secondary antibody, a FITC-conjugated anti-Rabbit was incubated for 1 hour at room temperature (Vector Laboratories, Inc, Burlingame, CA). For double labeling, TG2 and F4/80, we visualized antigen-antibody complexes with secondary antibodies conjugated with fluorochromes. Specimens were analyzed using a confocal microscope (Zeiss).

In Situ *End-Labeling for the Detection of Apoptotic Cells*

Fragmented nuclear DNA associated with apoptosis was labeled in histological sections with digoxigenin deoxyuridine, according to standard methods, using ApopTag Plus, peroxidase Kit (Qbiogen, France). After deparaffinization and dehydration, 4 μm-tissue sections were digested by incubation with 20 μg/ml proteinase K for 15 minutes at room temperature to enable the enzymatic permeabilization of the section for even incorporation of nucleotides, and then washed in distilled water four times for 2 minutes each wash. Endogenous peroxidase was inactivated by 3% (v/v) H_2O_2 in PBS for 5 minutes at room temperature. The sections were rinsed with PBS, immersed in TdT labeling buffer at 37°C for 15 minutes, and then incubated with TdT and digoxigenin-deoxyuridine at 37°C for 60 minutes. The reaction was terminated by transferring the slides to stop/wash buffer at room temperature for 10 minutes. The sections were washed in PBS three times for 5 minutes each wash. Then the slides were incubated with the anti-digoxigenin-conjugate for 30 minutes at room temperature and rinsed in PBS three times for 5 minutes each wash. The slides were developed by using peroxidase diaminobenzidine substrate Kit. The sections were counterstained in 0.5% (w/v) methyl green (Sigma) for 10 minutes at room temperature. The slides were mounted with permanent mounting media after washing with water, dehydrated with 100% *N*-butanol and cleared in xylene.

Total RNA Extraction and Quantitative Real Time PCR for COL1A2

Total RNA was extracted from renal cortex ($n = 6$ for all groups) using TRIzol reagent (Gibco BRL) and methyl trichloride according to the manufacturer's instructions. RNA quality was checked by control of optical density at 260 and 280 nm, and by electrophoresis. cDNA was synthesized from 1 μg of total RNA in 20 μl using a reverse transcriptase (Superscript II, Gibco BRL) as recommended by the manufacturer. Then, cDNA was amplified in a thermocycler (ABI Prism 7000) as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 45 seconds and

60°C for 1 minute, by using a Quantitect Probe PCR Kit (Qiagen) and TaqMan Gene expression assays (Applied Biosystems): TaqMan MGB probes, FAM dye-labeled of col 1 α 2 procollagen type I α 2 (Mm00483888_m1) and 18s (Hs99999901_s1) for normalization. Normal renal cortex cDNA was used as reference to establish calibration curves of col I and 18S CT (cycle threshold). All samples were assayed in triplicate, and the average value of the triplicate was used for quantification. Final results are expressed as the col I/18s cDNA ratio.

Kidney Collagen Content

Total renal collagen was measured by hydroxyproline assay as described previously.[22] In brief, an accurately weighed portion of the kidney was homogenized in 5 mmol/L $NaHCO_3$, 0.1 M NaCL solution, hydrolyzed in 10 N HCl, and incubated at 110°C for 18 hours. After acid neutralization, hydroxyproline released is oxidized with chloramines T at pH 6 and colored with 4-dimethylaminobenzyldehyde. Sample absorbance was measured at 550 nm. Total collagen in tissues was calculated on the assumption that collagen contains 12.7% hydroxyproline by weight. Final results were expressed as μg/mg kidney weight.

Determination of Tissue TGF-β1 and Monocyte Chemoattractant Protein 1 (MCP1) Concentrations by Enzyme-Linked Immunosorbent Assay

Renal concentrations of TGF-β1 and Monocyte chemoattractant protein 1 (MCP1) were determined using commercial kits (Quantikine TGF-β1 ELISA kit, and Quantikine Mouse JE/MCP-1 ELISA kit, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Kidney tissues were homogenized with 10 μl/mg extraction buffer (50 mmol/L HEPES, 50 mmol/L tetrasodium pyrophosphate, 100 mmol/L sodium fluoride, 10 ml EDTA, pH 7.4, 10 mmol/L Na-orthovanidate, and protease inhibitor cocktail). The concentration of active TGF-β1 protein was obtained from samples that were not acidified, whereas the levels of latent TGF-β1 protein were calculated by subtracting the active from total TGF-β1. The concentrations of TGF-β1 and MCP-1 in the kidneys were report to the weight of kidney tissue and expressed as pg/ml.

Statistical Analyses

Values are expressed as mean ± SD. Data were analyzed using one-way analysis of variance followed by protected least significant difference Fisher's test of the Stat view software package. Results with $P < 0.05$ were considered statistically significant.

Results

Tissue TG2-deleted Mice Have Normal Renal Phenotype

TG2 mice had been generated and described previously by De Laurenzi and Melino.[19] These mice did not show development abnormalities, and histological examination of the major organs appeared normal. Six-month-old male mice were studied in basal conditions ($n = 12$ per group). Tail cuff systolic arterial blood pressure and the heart rate were not different between groups, 123.9 ± 3.8 mm Hg and 122.2 ± 2.4, $P = 0.98$, 508 ± 27 and 482 ± 18 beat per minute, $P = 0.44$ for WT and KO mice, respectively. Both groups of mice had similar glomerular filtration rate measured by FITC-inulin clearance at 0.607 ± 0.04 ml/min/g of kidney in WT and 0.563 ± 0.08 ml/min/g of kidney in KO, $P = 0.63$. Urine albumin excretion was 1.97 ± 0.3 mg/mmol of creatininuria in WT mice and 3.12 ± 0.1 mg/mmol of creatininuria in KO mice, $P < 0.001$. The rates were different but still in the physiological range. At baseline, no specific renal histological lesions, in particular glomerular lesions were observed in control as in deficient mice (data not shown).

UUO Induces Renal Interstitial Fibrosis in WT Mice

Twelve days of UUO induced tubular dilation, renal cortical atrophy, cell infiltration, and ECM accumulation on Masson's trichrome staining (Figure 1A). Fibrillar collagen assessed by red Sirius staining with polarized light was increased after UUO (Figure 2A). Fibrillar collagen measured

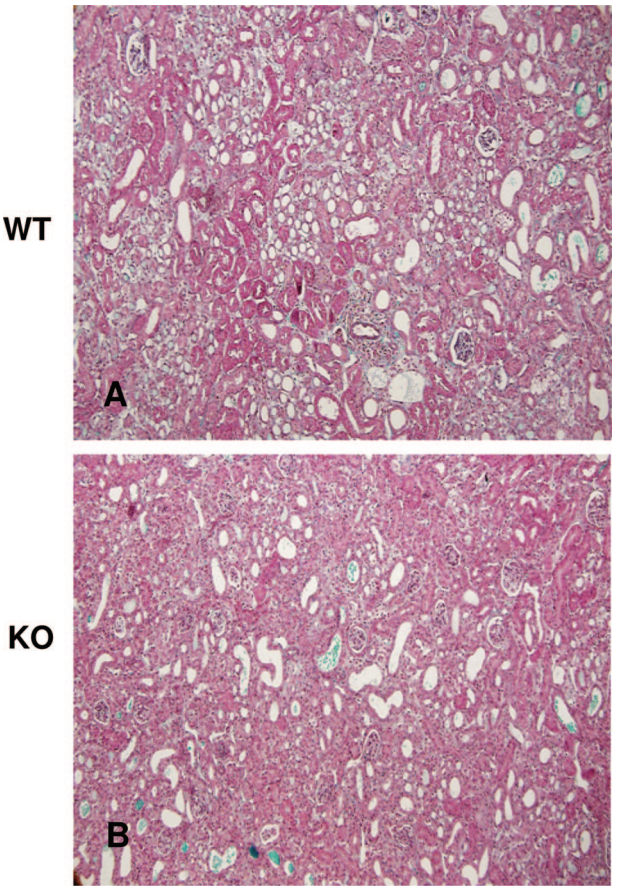
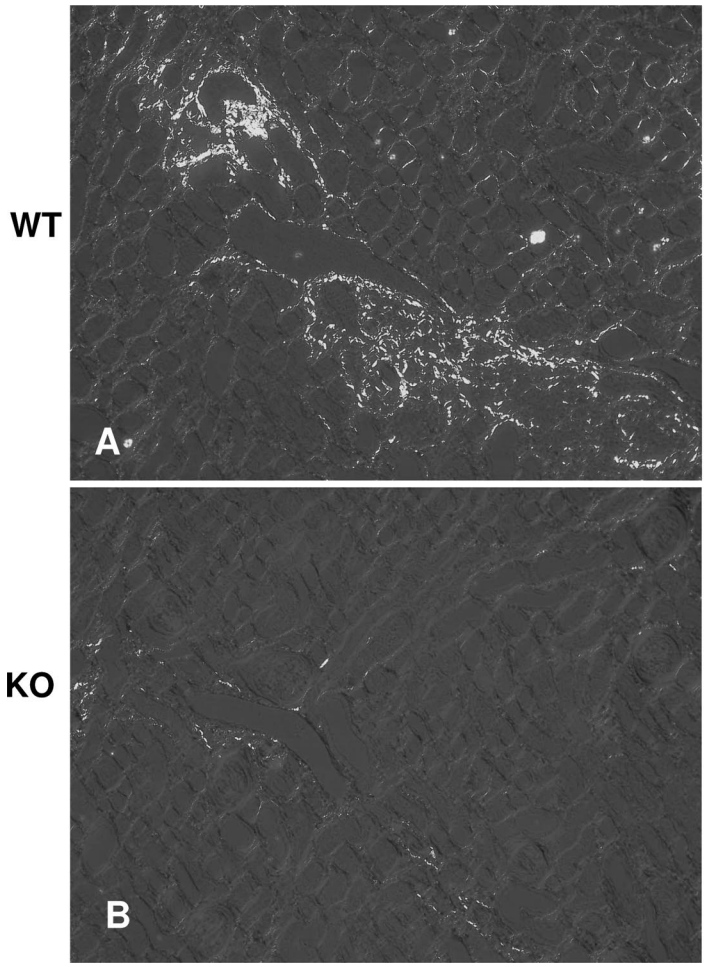


Figure 1. Representative example of renal tissue lesions on Masson's Trichrome staining. At baseline, no renal histological lesions were observed in control and KO mice (not shown). UUO induced tubular dilatation, renal cortical atrophy, cell infiltration and extracellular matrix accumulation in both WT (**A**) and KO mice (**B**).



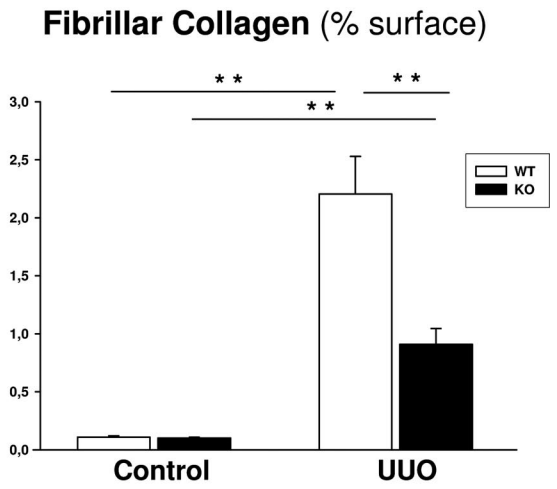


Figure 2. Representative example of renal fibrillar collagen content revealed by red Sirius staining and polarized light in WT (**A**) and TG2 KO mice (**B**), after UUO. Note the lower content of interstitial fibrillar collagen in TG2 KO mice compared to WT mice. Quantification by morphometric analysis on the right. Values are mean ± SEM; $n = 10$; $^{*}P < 0.05$; $^{**}P < 0.01$.

by morphometric analysis represented 0.108 ± 0.008% of the examined area at baseline, increased to 2.205 ± 0.325% after UUO, $P = 0.005$. Accordingly, total collagen content assessed by hydroxyproline assay was greater in UUO than in sham-operated mice (34.3 ± 7.3 vs. 14.7 ± 2.6 μg/mg of kidney, $P < 0.05$). Macrophage infiltration rose after UUO, from 0.49 ± 0.02% of examined area to 24.53 ± 3.49%, $P = 0.004$ in obstructed kidneys (Figure 3, A and B). According to previous studies, UUO induced an increase in S100A4-positive cells number, from 0.05 ± 0.01% to 2.38 ± 0.30%, $P < 0.001$ (Figure 4). S100A4-positive cells were visualized in both interstitium and tubules. Tubular S100A4-positive cells were also positive for megalin, which confirmed that they were localized in the proximal tubules where epithelial-mesenchymal transition occurs (Figure 5). Concomitantly with *de novo* expression of S100A4 myofibroblast marker, tubular E-cadherin staining totally disappeared after UUO (not shown). Renal MCP-1 concentration increased after UUO from 1.5 ± 0.5 to 1073.3 ± 176.8 pg/ml. Importantly, in WT mice, UUO induced a tremendous interstitial expression of TG2 that predominated in periglomerular and peritubular area, $P < 0.001$ (Figure 6B), whereas at baseline TG2 expression was faint and limited to peritubular capillaries (Figure 6A). As reported before, KO mice did not show any expression of TG2 (Figure 6, C and D).[19] Double staining of TG2 (red) and F4/80 (green) showed a partial colocalization suggesting that the TG2 overexpression originated from different cell types and was not exclusively from macrophage (Figure 7). There were 15-fold more apoptotic cells after UUO than in control conditions (0.89 ± 0.07% and 0.06 ± 0.01% respectively, $P < 0.001$). In WT mice, type 1 collagen deposits, which were barely detectable in sham-operated mice around the larger vessels, showed a significant rise after UUO, that represented respectively 0.9 ± 0.4 and 9.9 ± 2.7% of area examined by immunohistochemistry and morphometric analysis, $P < 0.01$ (Figure 8, A and B). Moreover, UUO induced type 1 collagen synthesis as shown by Col1A2 mRNA, which was 0.05 ± 0.02 at baseline as compared with 0.22 ± 0.03 after UUO, $P < 0.001$ (Figure 9). In agreement with previous studies, we observed a TGF-β1 increase in this UUO model. Total renal cortical TGF-β1 was 293 ± 5 pg/ml in sham-operated mice compared to 1514 ± 139 pg/ml after UUO, $P < 0.01$.

TG2 Deletion Protects from Renal Interstitial Fibrosis

Similar type of lesions were observed in KO and WT mice after UUO on Masson trichrome staining (Figure 1, A and B). In KO mice, UUO induced fibrillar collagen deposits that increased from 0.10 ± 0.02 to 0.91 ± 0.13 positive area, but

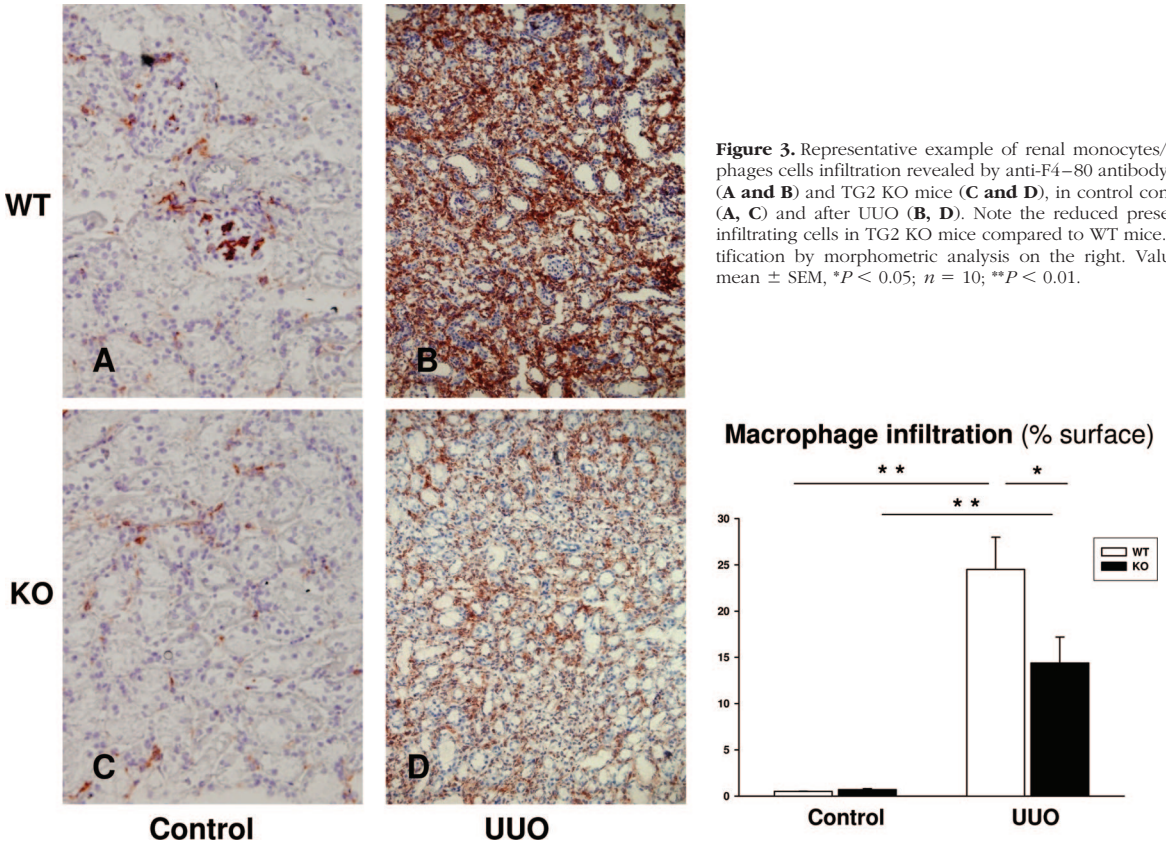


Figure 3. Representative example of renal monocytes/macrophages cells infiltration revealed by anti-F4–80 antibody in WT (**A and B**) and TG2 KO mice (**C and D**), in control conditions (**A, C**) and after UUO (**B, D**). Note the reduced presence of infiltrating cells in TG2 KO mice compared to WT mice. Quantification by morphometric analysis on the right. Values are mean ± SEM, $^{*}P < 0.05$; $n = 10$; $^{**}P < 0.01$.

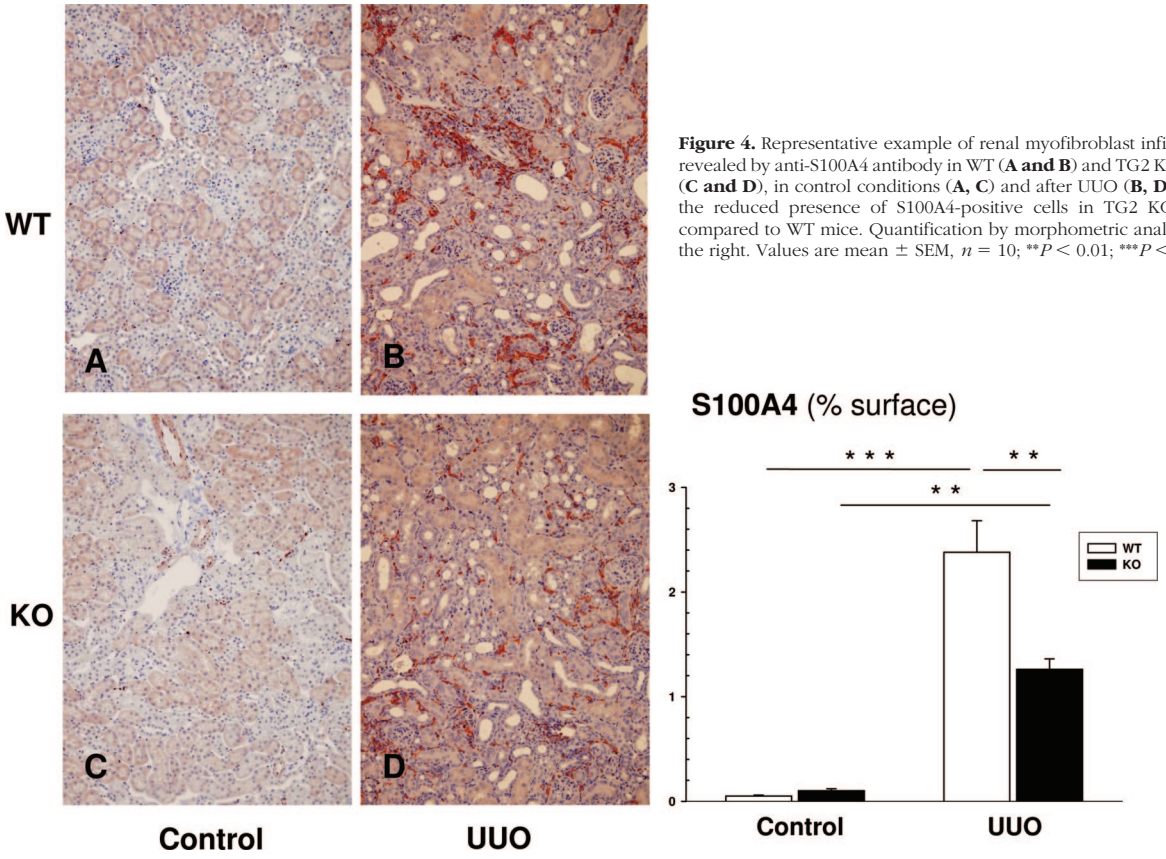


Figure 4. Representative example of renal myofibroblast infiltration revealed by anti-S100A4 antibody in WT (**A and B**) and TG2 KO mice (**C and D**), in control conditions (**A, C**) and after UUO (**B, D**). Note the reduced presence of S100A4-positive cells in TG2 KO mice compared to WT mice. Quantification by morphometric analysis on the right. Values are mean ± SEM, $n = 10$; $^{**}P < 0.01$; $^{***}P < 0.001$.

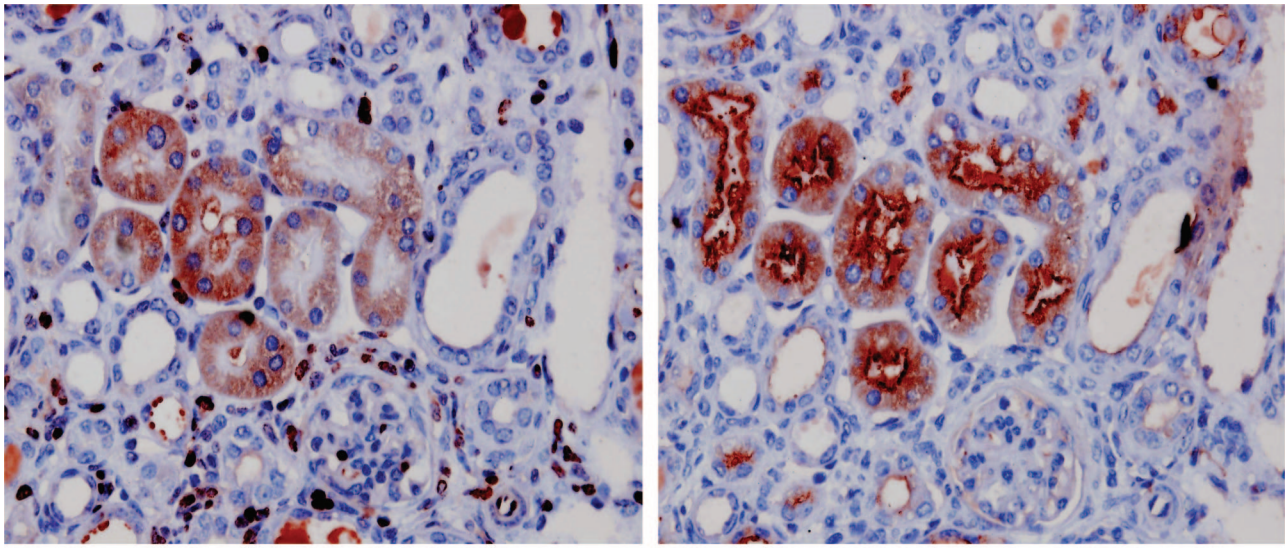


Figure 5. Localization of S100A4-positive cells and proximal tubular cells revealed by megalin antibody, in serial sections from WT UUO mice. Tubular S100A4-positive cells were positive for megalin, demonstrating that they belonged to the proximal tubules.

to a lesser extent than in WT UUO mice, $P = 0.005$ (Figure 2B). Total collagen content in KO mice at baseline was 16.4 ± 3.3 μg/mg of kidney and did not change significantly after UUO (22.7 ± 3.8 μg/mg of kidney, $P = 0.21$) as type 1 collagen deposit, $P = 0.94$ (Figure 8C–D). Similarly, type 1 collagen synthesis did not reach a significant increase (Figure 9). Altogether, our results show that ECM protein deposition, more specifically type 1 collagen (a major component of fibrosis), was substantially reduced in KO mice as compared with WT mice. In addition, S100A4-positive cells number increased in KO mice from 0.10 ± 0.02% to 1.26 ± 0.10% after UUO, $P < 0.001$, but to a lesser extend than in WT mice, $P < 0.01$ (Figure 4). E-cadherin staining persisted weakly in KO mice after UUO. Likewise, the macrophage infiltration after UUO in KO mice was milder than in WT mice, $P < 0.05$ (Figure 3C–D). Renal MCP-1 concentration increased from 2.6 ± 1.66 to 1140.1 ± 209.4 pg/ml after UUO, as in WT mice. The number of apoptotic cells rose in KO mice after UUO from 0.07 ± 0.02% to 1.06 ± 0.21%, $P < 0.01$, but it was not different from WT mice. These results show that, TG2 deficiency markedly protects from development of interstitial renal fibrosis after UUO.

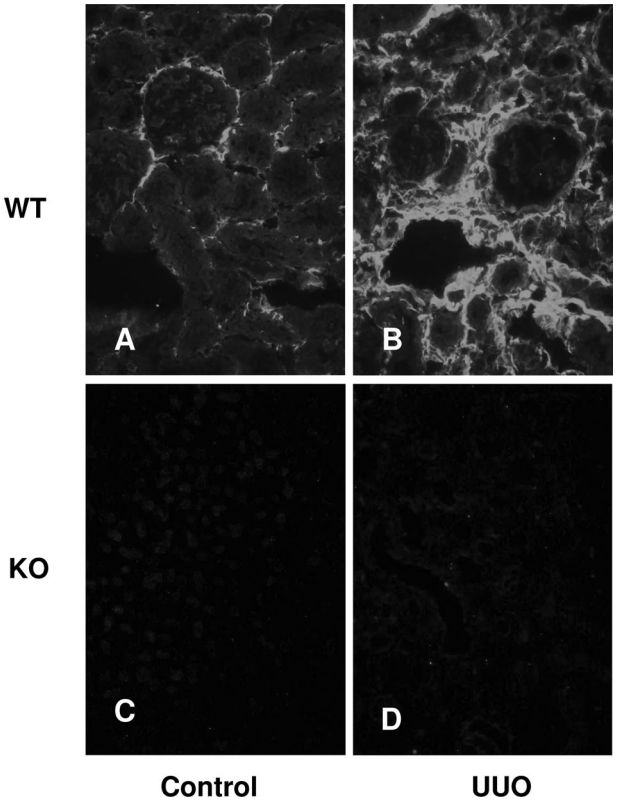


Figure 6. Renal TG2 expression revealed by anti-tissue transglutaminase antibody, in control conditions (**A**) and after UUO (**B**) in WT mice. TG2 KO mice do not express TG2 (**C** and **D**). In WT mice, TG2 is expressed in glomeruli and in intertubular vessels at baseline. Note the periglomerular and interstitial increased expression of TG2 induced by UUO in WT mice.

TG2 Contributes to Renal Fibrosis through TGF-β1 Activation

To understand the mechanism that explains the milder interstitial renal fibrosis and lower deposit and synthesis of type 1 collagen in KO relative to WT mice, we focused on TG2 profibrotic actions. Previously, TG2 has been shown to confer matrix resistance to degradation through covalent γ-glutamine-lysine bonds between different ECM proteins.[8] Moreover, TG2 contributes to TGF-β1 activation by favoring the binding of latent TGF-β binding protein-1 to ECM, which is a prerequisite step to TGF-β1 release. We assessed TGF-β1 activation in renal cortex. In both groups, UUO induced a similar increase of total TGF-β1 reaching 1514 ± 139 and 1421 ± 312 pg/ml in WT and KO UUO mice, respectively. However, active TGF-β1 was twice lower in renal cortex of KO mice than

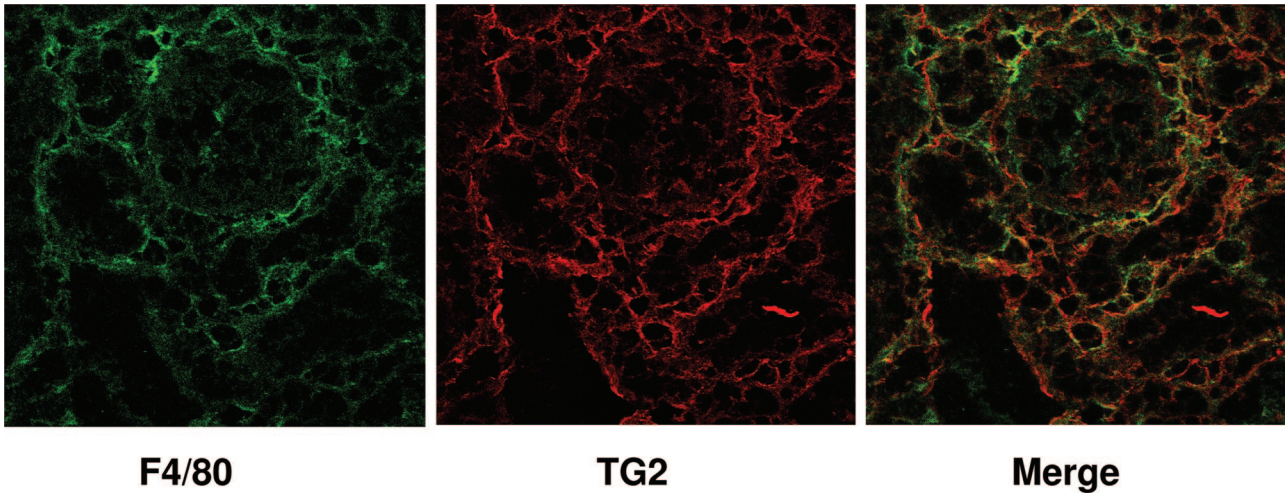


Figure 7. Localization of macrophages and TG2 using a double staining of F4/80 (green) and TG2 (red) in WT mice after UUO. Both pattern of staining were mainly interstitial but colocalization was only partial.

WT mice at 117 ± 17 and 249 ± 46 pg/ml, respectively (Figure 10).

Discussion

In this study, we have investigated for the first time the role of specific inhibition of TG2 in interstitial renal fibrosis using KO mice and explored the pathway of TG2 profibrotic action. Previous studies in experimental models and in human nephropathies showed a correlation between TG2 expression and the development of interstitial and glomerular sclerosis. *In vitro* results suggested a profibrotic action of TG2 because of its capacity to crosslink ECM proteins, thus increasing their resistance to degrada-

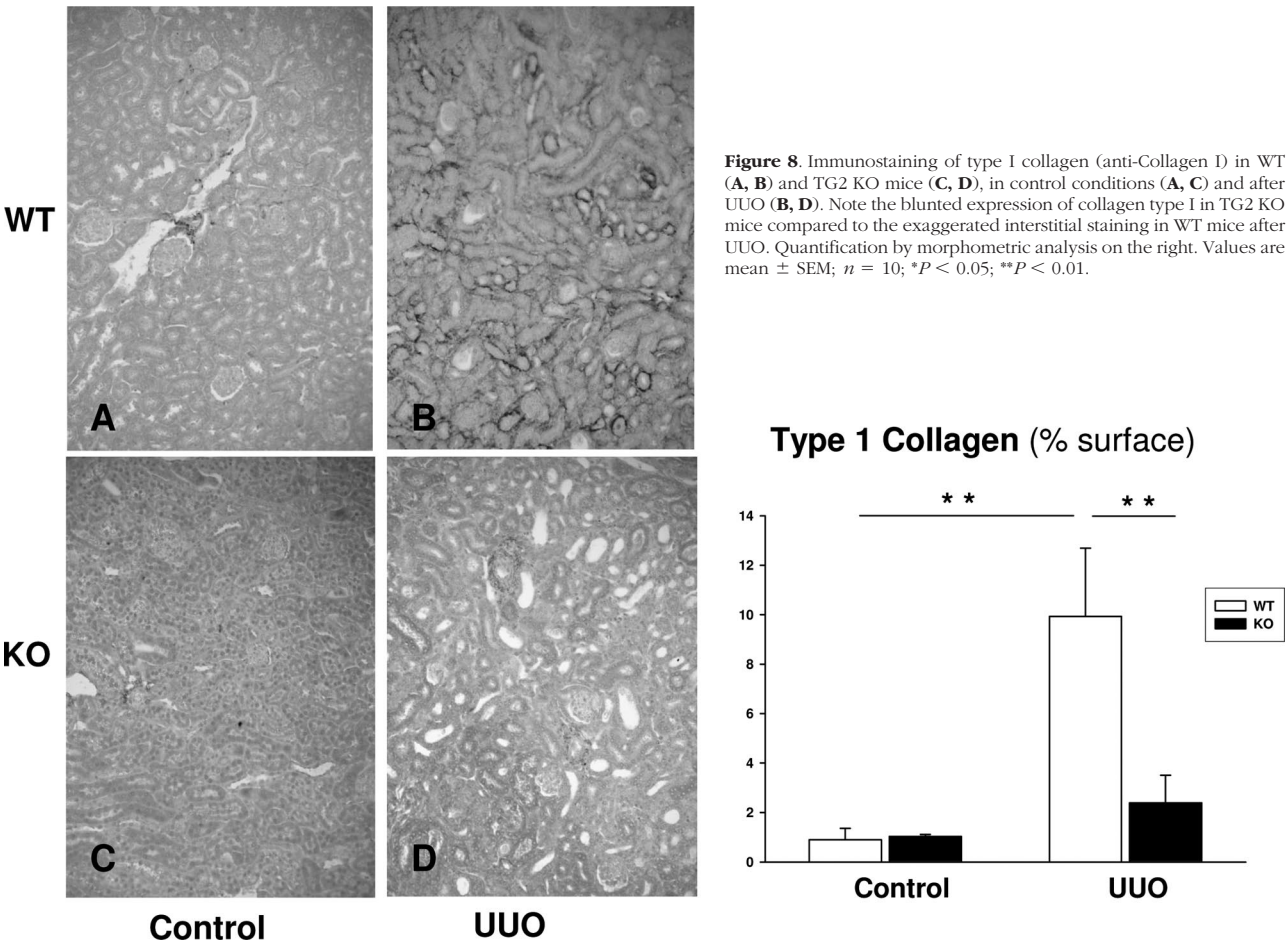


Figure 8. Immunostaining of type I collagen (anti-Collagen I) in WT (**A, B**) and TG2 KO mice (**C, D**), in control conditions (**A, C**) and after UUO (**B, D**). Note the blunted expression of collagen type I in TG2 KO mice compared to the exaggerated interstitial staining in WT mice after UUO. Quantification by morphometric analysis on the right. Values are mean ± SEM; $n = 10$; $^{*}P < 0.05$; $^{**}P < 0.01$.

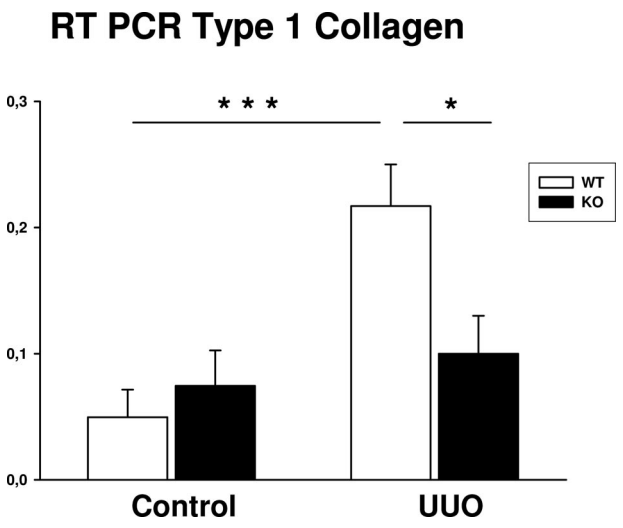


Figure 9. Quantitative RT-PCR of alpha2 collagen I in WT and TG2 KO mice, in the renal cortex under control conditions and after UUO. Note the absence of α2ColI mRNA increase in KO mice compared to WT mice induced by UUO. Values are mean ± SEM; $n = 8$; $^{*}P < 0.05$; $^{***}P < .01$.

tion. These data were recently confirmed *in vivo* in rats with the five-sixths nephropathy model that were treated with transglutaminase pharmacological inhibitors.[14] However, it is likely that these inhibitors target all TG, with varying potencies, including factor XIII, which like TG2, is associated with ECM, and seems to be involved in early stages of wound repair.[23] Our objective was to understand the specific role of TG2, irrespective to other TGs in progression of renal disease in an inflammatory model. To this end, we applied a model of obstructive nephropathy in mice lacking expression of TG2 and we observed that they were protected against the development of fibrotic and inflammatory lesions in the renal interstitium. In addition, we found that TG2 contributed to renal fibrosis by increasing myofibroblasts and collagen I synthesis through a mechanism involving TGF-β activation. Our results provide novel *in vivo* evidence about the profibrotic role of TG2 in renal disease and show the therapeutic importance of targeting TG2 activity.

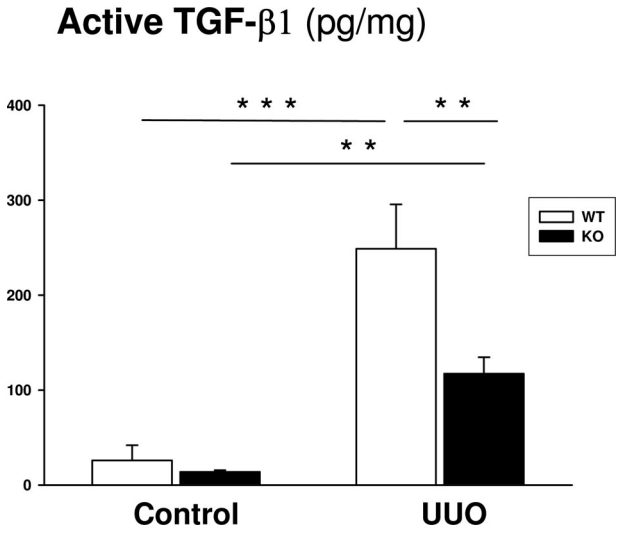


Figure 10. Concentration of active TGF-β1 concentration in the renal cortex of WT and TG2 KO mice, under control conditions and after UUO. Note the lesser levels of active TGF-β1 concentration in KO mice after UUO compared to WT mice. Values are mean ± SEM; $n = 10$; $^{**}P < 0.05$; $^{***}P < .01$.

We chose to investigate the UUO model of renal interstitial fibrosis because TG2 exerts its profibrotic effects mainly on interstitial ECM. In control conditions, we found a faint TG2 expression in peritubular capillaries in agreement to previous reports.[17] This pattern of expression dramatically changed after UUO where TG2 was strongly induced in the renal interstitial compartment, as also observed in experimental and human nephropathies. In the rat streptozotocin model, TG2 expression and activity were increased predominantly in the peritubular interstitial space and correlated closely to the expression of interstitial collagen.[24] Likewise, increased periglomerular, mesangial, and interstitial staining for TG2 was observed in 136 human biopsies from a range of chronic kidney diseases, mostly glomerular diseases.[15] In human IgA nephropathy, TG2 staining was observed mainly in interstitial fibrotic area.[17] During chronic allograft nephropathy, the elevation of TG2 expression was correlated with interstitial fibrosis.[16] In addition, several studies showed that the index of interstitial fibrosis was correlated to increased TG2 activity. The interstitial expression of TG2 is likely due to the increased externalization of the enzyme from epithelial cells through a mechanism still incompletely understood.[8,24] The externalization of TG2 would facilitate its activation because of the high extracellular calcium concentration compared to intracellular level. In our experiments, the only partial colocalization between macrophage and TG2 positive cells suggest the contribution of renal resident cells in TG2 overexpression. Because UUO-induced nephropathy is characterized by epithelial-mesenchymal transition, it is possible that changes in the phenotype of tubular proximal epithelial cells are involved in TG2 over-expression. In this regard, it has been reported *in vitro* that proximal tubular epithelial cells exposed to elevated glucose displayed an mRNA-dependent increase in TG2 activity with concomitant increase of ECM synthesis and ECM $\varepsilon(\gamma$-glutamyl) lysine cross linking.[25] Our observations provide further evidence that the induction of TG2 expression in the renal interstitium is a common event of the development of renal disease, irrespective of the initiating cause.

The fact that TG2 is overexpressed in chronic renal disease does not necessarily mean that this overexpression is involved in the development of renal fibrosis. If our hypothesis for a profibrotic action of TG2 is correct, then TG2 inhibition should confer protection. We decided to use a genetic rather than a pharmacological approach for the following reasons. First, cystamine, which inactivates TG2 activity by forming a mixed disulfide, may interfere with and inhibit other thiol-dependent enzymes such as caspases.[26] Second, other inhibitors including imidazolium derivatives also target other transglutaminases such as factor XIII.[27] We showed that mice with genetically inhibited expression of TG2 were protected against the development of fibrotic lesions in obstructive nephropathy. This result suggests that TG2 over-expression is a pivotal mediator of progression of renal failure. Very recently, Johnson et al[14] showed that administration

of pharmacological inhibitors of TG2 reduced the development of glomerulosclerosis and interstitial fibrosis induced by 5/6-nephropathy, although the mechanism of prevention was different (see below).

To explain the profibrotic role of TG2 we initially hypothesized that TG2 would act by stabilizing the exaggerated ECM formation through its crosslinking property. However, we observed that TG2 null mice displayed a lower myofibroblast infiltration and a reduced rate of collagen I synthesis after UUO as evidenced by the blunted decrease of mRNA expression, protein expression, and protein content of collagen I, in the renal tubular interstitium. These results suggested that TG2 activation could lead to increased myofibroblast number and collagen I synthesis. Because TGF-β is a major mediator of increased collagen synthesis and epithelial-mesenchymal transition, we examined whether the lack of TG2 affected expression and/or activity of TGF-β. TGF-β involvement in these fibrogenic mechanisms has been well established in several experimental models including UUO.[3,28–30] We found a fivefold increase of total (latent + active) TGF-β1 concentration in both, WT and KO mice after UUO, but the increase of active TGF-β1 following UUO was blunted in mice lacking the expression of TG2. Under normal conditions, latent TGF-β1 procytokine-complexes and TGF-β1 receptors are widely expressed in most tissues, whereas little active form of TGF-β1 is present in tissues, supporting the concept of critical regulation of TGF-β1 action by its activators, which has been confirmed in a series of experiments. *In vivo*, gene transfer of the constitutively active TGF-β gene into the lung of rats caused extensive fibrosis while overexpression of the latent TGF-β transgene did not.[31] In several pathophysiological conditions the increased action of TGF-β results from a post-transcriptional regulation of transforming latent TGF-β1 into active. UUO may facilitate activation of latent TGF-β1 through several pathways that may involve generation of plasmin from plasminogen by t-PA, matrix metalloproteinases-2 and -9,[32] thrombospondin-1,[33] or $\alpha_v\beta_6$ integrin,[34] and TG2.[35] Interestingly, it has been shown that latent TGF-β binding protein-1 and large complex are substrates for TG2.[35,36] Matrix incorporation of large latent complex, which is a mandatory step to active TGF-β1 release, is TG2 dependent. In addition, TGF-β1 induces TG2 transcription as isolated perfused kidney with human recombinant TGF-β1 showed an eightfold increase in TG2 mRNA in renal cortex.[37,38] We propose that the lesser concentration of active TGF-β1 in TG2 deficient mice, accounted for the lower myofibroblast activation, the decreased collagen I synthesis, and the relative protection of those mice against the UUO-induced fibrosis. In agreement with our hypothesis, reduction of active TGF-β1 either by antisense treatment or via thrombospondin-1 inhibition decreased collagen 1 synthesis, ECM accumulation, and alleviated renal fibrosis in UUO nephropathy.[39,40] Johnson et al failed to detect any reduction in either active or total TGF-β1 in the five-sixths nephrectomy model in rats treated with TG inhibitors.[14] This discrepancy may be due to the different models used or to other effects of TG pharmacological inhibitors that wound counterbalance the TG2 effects on TGF-β1 activation.

In addition to renal interstitial ECM deposit, the UUO model is also characterized by increased cell apoptosis and an accumulation of monocytes/macrophages that contributes to renal injury.[41] The absence of difference in the level of apoptosis between the groups, in both control conditions and after UUO, suggests a weak effect of TG2 on renal cell apoptosis in this model. In contrast, we showed that TG2 deficiency reduced monocyte/macrophage infiltration. A reduction in interstitial cell numbers was also observed in rats treated with pharmacological TG2 inhibitors.[14] The molecular basis of cell recruitment is a complex process that involves chemoattraction and cell adhesion events. TG2 has been involved in nuclear factor kappa-B activation through cross-linking of I-kBα a mechanism independent of I-kB kinase.[42] In turn, nuclear factor kappa-B seems to play a key role in UUO by inducing MCP-1 and regulated on activation normal T cell expressed and secreted (RANTES).[43,44] The similar increase of renal MCP-1 concentration in both TG2-deficient and WT mice does not support an effect of TG2 on nuclear factor kappa-B pathway. One possible explanation is the role of TG2 in the adhesion and migration of monocytes independently of its catalytic action. *In vitro*, inhibition of cell surface TG2 expression decreased adhesion and migration of monocytic cells on fibronectin.[45] An alternative hypothesis is the lower TGF-β activation itself. Actually, mice lacking smad3, a key signaling downstream of TGF-β receptors were protected against tubulointerstitial fibrosis following UUO with abrogation of monocyte influx and collagen accumulation.[30] The reduced monocyte/macrophage infiltrate observed in TG2 deficient mice strongly supports a role for TG2 in the renal inflammation characteristic of UUO, and most likely contributes together with the decreased active TGF-β1 in the prevention of renal fibrosis. Further studies are needed to unravel the mechanisms whereby TG2 increases interstitial cell inflammation in UUO and others models.

In conclusion, our results show that TG2 plays an important role in the development of renal fibrosis. During early stages of fibrosis in obstructive nephropathy, as is the case of the present investigation, TG2 favors TGF-β1 activation, cell infiltration, epithelial to mesenchymal transition, and ECM accumulation. It is possible that in other models and/or in late stages of established fibrosis, TG2 increases the resistance of ECM proteins to degradation. Our results provide evidence of a beneficial effect of inhibiting TG2 to limit renal scarring. Targeting TG2 expression and/or activation can provide a novel and important additional treatment to combat renal fibrosis.

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